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## Two Tumor Necrosis Factor-binding Proteins Purified from Human Urine

EVIDENCE FOR IMMUNOLOGICAL CROSS-REACTIVITY WITH CELL SURFACE TUMOR NECROSIS FACTOR RECEPTORS\*

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Hartmut Engelmann, Daniela Novick, and David Wallach†

From the Department of Molecular Genetics and Virology, The Weizmann Institute of Science, Rehovot 76100, Israel

Two proteins which specifically bind tumor necrosis factor (TNF) were isolated from human urine by ligand (TNF)-affinity purification, followed by reversed phase high performance liquid chromatography. The molecular weights of the two proteins, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, were similar (about 30,000). Both proteins provided protection against the cytotoxic effect of TNF *in vitro* and both bound TNF- $\alpha$  more effectively than TNF- $\beta$ . Antibodies raised against each of the proteins had an inhibitory effect on the binding of TNF to cells, suggesting that both proteins are structurally related to the TNF receptors. However, the two proteins differed in NH<sub>2</sub>-terminal amino acid sequences: Asp-Ser-Val-Cys-Pro- in one and Val-Ala-Phe-Thr-Pro- in the other. The NH<sub>2</sub>-terminal sequence of the former protein was invariable, while that of the latter was truncated to varying degrees. The two proteins were also immunologically distinct. The relative efficacy of antisera against the two proteins in inhibiting the binding of TNF to cells varied markedly from one line of cells to another. Evidence has been presented recently for the existence of two distinct molecular species of cell surface receptors for TNF and for differential expression of those two receptors by cells of different lines. The findings presented in this study are consistent with the notion that the urinary TNF-binding proteins constitute soluble forms of the two molecular species of the cell surface TNF receptors.

Effects of tumor necrosis factor (TNF)<sup>1</sup> on cell function contribute, in a number of ways, to the defense of the organism against infectious agents and to recovery from injury. However, in certain pathological situations, some of these same effects may become detrimental, causing even more harm than the pathogen which induced the formation of TNF (1,

2). There is therefore particular interest in elucidating the mechanisms whereby these effects are regulated.

In the sequence of molecular events that take place in the response of the cell to TNF, the most readily accessible to modulation is the initiation of this process, triggered by the binding of TNF to its cell surface receptors. One way of modulating this interaction is by inducing changes in the expression of the TNF receptors. Both the number and affinity of the receptors were found to vary in response to certain regulators (3-7). Furthermore, there may be also variation in the kind of receptor molecules which cells can express. Recent studies suggest that there are two different molecular species of TNF receptors and that these are expressed differentially by cells of different lines.<sup>2</sup>

A different way of modulating the binding of TNF to its receptors was reported in several recent studies, in which a protective effect of proteins found in human urine against the cytotoxic activity of TNF was described (8-10). This protection could be related to the function of certain minor constitutive proteins of the urine which bind TNF and thus decrease its availability to the TNF receptors. One of these proteins has been purified (10-12). Here, we present evidence that there are two different TNF-binding proteins in the urine. Evidence is also presented suggesting that these proteins may be structurally related to the two cell surface TNF receptors. These findings raise the possibility that cells can produce receptors for TNF also in a soluble form and that, when presented in such form to the cell, the receptors can compete for TNF with the membrane-associated receptors and thus function as inhibitors for TNF activity.

### MATERIALS AND METHODS

#### Cells

Cells of the HeLa (13), MCF7 (14), K562 (15), and U937 (16) lines were cultured in RPMI 1640 medium supplemented with 100  $\mu$ g/ml penicillin, 100  $\mu$ g/ml streptomycin, and 10% heat-inactivated fetal calf serum.

#### Cytokines

The recombinant human TNF- $\alpha$  (rhuTNF- $\alpha$ ,  $3.2 \times 10^7$  units/mg protein) used for the affinity purification of the TNF-binding proteins was purchased from Pharma Biotechnologie, Hanover, West Germany. The rhuTNF- $\alpha$  used for all other purposes ( $6 \times 10^7$  units/mg protein) and the recombinant human TNF- $\beta$  (rhuTNF- $\beta$ , lymphotoxin,  $1.2 \times 10^8$  units/mg protein) were kindly provided by Dr. G. Adolf of the Boehringer Institute, Vienna, Austria. Recombinant human IL-1 $\alpha$  (rIL-1 $\alpha$ ,  $2.5 \times 10^9$  units/mg protein), consisting of the

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† To whom correspondence and reprint requests should be addressed.

<sup>1</sup> The abbreviations used are: TNF, tumor necrosis factor; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; TBP, TNF-binding protein; SDS, sodium dodecyl sulfate; IL-1, interleukin-1; IL-6, interleukin-6; PBS, phosphate-buffered saline; r, recombinant; hu, human; BSA, bovine serum albumin.

<sup>2</sup> H-P. Hohmann, R. Remmy, M. Brockhaus, and A. P. G. M. Van Loon, an abstract presented in the 2nd International Conference on TNF and Related Cytokines, Napa, CA, January 15-20, 1989; and M. Brockhaus, H. Loetscher, H-P. Hohmann, and W. Huziker, *ibid*.

1531 and acknowledgements

154 carboxyl-terminal amino acids of the 271-amino acid human IL-1 precursor, was a gift of Drs. A. Stern and P. T. Lomedico (Hoffman-La Roche, Nutley, NJ). Recombinant human interferon- $\gamma$  (rIFN- $\gamma$ ,  $5 \times 10^7$  units/mg protein) and recombinant human interleukin 6 (rIL-6,  $3 \times 10^6$  units/mg protein, as determined by the hybridoma growth factor assay) were a gift from InterPharm Laboratories, Ness Ziona, Israel. Radiolabeling of TNF- $\alpha$  (to 140  $\mu$ Ci/ $\mu$ g), TBPI (to 170  $\mu$ Ci/ $\mu$ g), and TBPII (to 290  $\mu$ Ci/ $\mu$ g) was done by the chloramine-T method, as described previously (4).

#### Antibodies

Three-month-old rabbits (New Zealand White) were immunized with TBPI and TBPII, at doses of 5 and 20  $\mu$ g, respectively, according to the following protocol: The proteins were first injected subcutaneously as emulsion in complete Freund's adjuvant. After 3 weeks, the animals were injected intramuscularly with an emulsion of the proteins in incomplete Freund's adjuvant and then twice, subcutaneously, at 1-week intervals with the proteins in PBS solution.

#### Assays for the TNF-binding Proteins

**Bioassays**—Quantitation of the protective effect of the TNF-binding proteins against TNF cytotoxicity and of their inhibitory effect on the binding of radiolabeled TNF to cells was performed as described before (10). When using suspended cells (K562 and U937), washing of unbound radiolabeled TNF was achieved by repeated centrifugation (6). All assays were performed in duplicates.

**Binding of TBP to Cytokines**—The solid phase assay for the binding of various cytokines to the purified TNF-binding protein was performed as described before (10).

#### Assays for Antibodies against TNF-binding Proteins

**Western Blotting Analysis**—Proteins to be tested were applied to SDS-PAGE (12% acrylamide gels, 1 mm thickness) and then blotted electrophoretically onto a nitrocellulose sheet (Schleicher & Schell, F.R.G.), using the Bio-Rad mini-protein II dual slab cell and mini-trans-blot devices. The nitrocellulose sheet was incubated for 2 h with 10% milk (v/v) in PBS containing 0.1% sodium azide and 0.05% Tween 20 and was then briefly rinsed in PBS containing 0.05% Tween 20 (PBS-Tween). It was then incubated for 2 h with the test antibodies in a multilane device followed by incubation for 1 h with  $^{125}$ I-labeled protein A (Amersham Corp., 40  $\mu$ Ci/ $\mu$ g,  $5 \times 10^5$  cpm/ml). After washing off the unbound material, the nitrocellulose sheet was exposed to autoradiography. All incubations were carried out at room temperature. All reagents were applied in 10% milk in PBS containing sodium azide and Tween 20, as above.

**ELISA**—Ninety-six-well ELISA plates (Nunc, Denmark) were coated with 1  $\mu$ g/ml of TBPI, TBPII, or BSA as a control, in PBS containing 0.02% sodium azide, by incubating the plates for 2 h at 37 °C followed by overnight incubation at 4 °C. The plates were then rinsed with PBS-Tween and incubated for 2 h with a solution of 0.5% BSA in PBS-Tween. After rinsing again with PBS-Tween the wells were incubated for 2 h with the test antibodies, then rinsed again and incubated for 2 h with purified goat antibody to rabbit IgG, conjugated to horseradish peroxidase (Biomakor, Israel). After a further washing step with PBS-Tween, the activity of horseradish peroxidase-conjugated antibody which bound to the plate was determined using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as a substrate. The enzymatic product was determined colorimetrically at 600 nm. All incubations were performed at 37 °C.

**Effect of the Antibodies on Binding of TNF to Cells**—The antisera to TBPI and TBPII were diluted in PBS containing 0.5% BSA and 0.1% sodium azide and then added either directly or after incubation for 30 min at 4 °C with a sample of TBP (Fig. 7) for 2 h to the test cells. The cells were then rinsed and tested for binding of TNF as described elsewhere (6).

#### Purification of the TNF-binding Proteins

**Ligand (TNF)-affinity Purification**—Proteins of pooled urine of healthy postmenopausal women were concentrated 600-fold as described before (10). A sample of 250 ml of the concentrate was applied at a flow rate of 0.25 ml/min to a column constructed of 0.5 ml of Affi-Gel 10 (Bio-Rad) to which 3.5 mg of rhuTNF- $\alpha$  (Pharma Biotechnology, Hannover) was coupled. The column was then washed with PBS until all unbound proteins were removed, and the bound proteins were eluted by applying a solution containing 25 mM citric acid, 100 mM NaCl, and 0.02% sodium azide, at pH 2.5.

**Reverse Phase HPLC**—The proteins eluted from the TNF affinity column were applied to an Aquapore RP300 column (4.6  $\times$  30 mm, Brownlee Labs) which was pre-equilibrated with 0.3% aqueous trifluoroacetic acid. The column was washed with the above solution at a flow rate of 0.5 ml/min until all unbound proteins were removed, and then eluted with a gradient of acetonitrile concentrations in 0.3% aqueous trifluoroacetic acid, as described before (10). Fractions of 0.5 ml were collected.

**SDS-PAGE**, visualization of proteins on the acrylamide gel by silver staining, NH<sub>2</sub>-terminal sequence analysis, and protein determinations were performed as specified before (10).

#### RESULTS

In a prior study we described the isolation of a protein from human urine that could protect cultured cells from the cytotoxic effect of TNF. The protein was purified to homogeneity in a series of chromatographic steps. It was found to bind TNF (10). Since the recovery of bioactivity in that procedure was low, the extent to which this protein contributes to the total protective activity of the unfractionated preparation of urinary proteins could not be determined. Therefore we attempted to isolate the protein in a more direct way, by affinity purification on a column of immobilized TNF.

Application of crude urinary proteins on such a column resulted in complete depletion of their protective activity, suggesting that this activity is fully mediated by TNF-binding protein(s) (Fig. 1 and Table I). The proteins which bound to the column could be eluted by decreasing the pH. The specific activity of the eluted proteins was about 20,000-fold higher than that of the crude urinary proteins. In SDS-PAGE analysis most of the proteins in the eluate migrated as a single broad band with apparent molecular size of  $30,000 \pm 2,000$  (Fig. 2A). The affinity-purified proteins were further fractionated by reversed phase HPLC. The proteins were applied to an Aquapore RP300 column and eluted with a gradient of acetonitrile concentrations in the presence of 0.3% trifluoroacetic acid. They resolved into two active components; one (TBPI), eluting at 27% acetonitrile, similarly to what was reported previously (10, 11). The second protein (TBPII) eluted at a somewhat higher concentration of acetonitrile (31%) (Fig. 3).

Both proteins had a protective effect against TNF cytotoxicity although the specific activity of TBPII was lower than that of TBPI (Fig. 1 and Table I). The binding activities of TBPI and TBPII were examined in a solid phase assay using radiolabeled preparations of the proteins. Both were found to

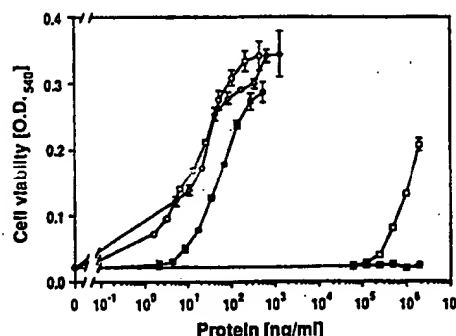


FIG. 1. Titration of the bioactivity of the TNF-binding proteins at differing stages of purification.  $\square$ , unfractionated urinary proteins;  $\blacksquare$ , effluent of the ligand (TNF)-affinity column;  $\circ$ , eluate of the ligand (TNF)-affinity column;  $\circ$ , pooled fractions of TBPI following HPLC (fractions 19–21 in the experiment depicted in Fig. 3);  $\bullet$ , pooled fractions of TBPII following HPLC (fractions 24–27, Fig. 3). Purification of the TNF-binding proteins and quantitation of their protective effect against the cytotoxicity of TNF were carried out as described under "Materials and Methods."

TABLE I  
Purification of the two TNF-binding proteins

Purification step	Protein	Protective activity		Specific activity	Purification
	mg	units <sup>a</sup>	%	units/mg	-fold
Crude urinary proteins	9,400	117,200	100	12.4	
Affinity purification					
Flow-through	9,300	Not detectable			
Eluted proteins	0.36	98,600	84.2	273,800	22,000
Reversed phase HPLC					
Total	0.217	31,200	26.6	143,400	11,600
TBPI	0.070	22,300	19.0	318,600	
TBPII	0.147	8,900	7.6	60,700	

<sup>a</sup> A unit of protective activity was defined as the amount of TNF-binding proteins in whose presence the number of cells remaining viable, under the conditions of the assay for the protective effect of the proteins against TNF cytotoxicity, was doubled (10).

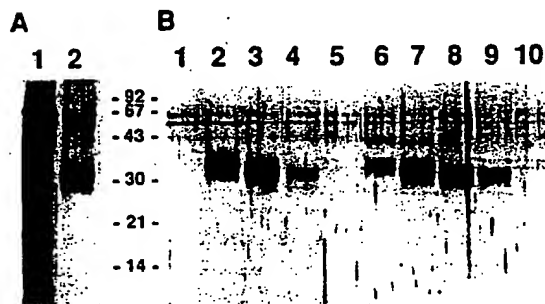


FIG. 2. SDS-PAGE analysis of the crude and purified preparations of the TNF-binding proteins. A, affinity purification step: 1, unfractionated urinary proteins; 2, proteins eluted from the TNF column by the low pH buffer. B, purification by reverse phase HPLC: 1-4, TBPI (fractions 18-21 in the experiment of Fig. 3); 5-9, TBPII (fractions 23-27); 10, sample buffer. Migration of molecular weight markers (Pharmacia) is also depicted. Analysis was made under reducing conditions. The two bands migrating somewhat further than the 67 kDa molecular mass marker, which are particularly prominent in lane 2, are also detectable in lane 10, where only sample buffer was applied. They reflect the presence of some contaminants in the  $\beta$ -mercaptoethanol.

bind TNF- $\alpha$  specifically. Excess TNF- $\alpha$  or TNF- $\beta$ , although less effectively, competed for this binding. Several other cytokines (IL-1, IL-6, IFN- $\gamma$ , Table II) did not compete. In SDS-PAGE analysis both TBPI and TBPII exhibited heterogeneity in molecular size, comparable to that observed for the protein applied on the reverse phase column. Some variation in molecular size, from one eluted fraction to another, could be observed (Fig. 2B).

In spite of this variation in molecular size, NH<sub>2</sub>-terminal microsequence analysis of TBPI revealed just a single NH<sub>2</sub>-terminal sequence: Asp-Ser-Val-Cys-Pro-, identical to that reported for this protein before (10, 11). On the other hand, in TBPII, a number of NH<sub>2</sub>-terminal sequences, all different from that of TBPI, could be observed. Furthermore, the quantitative proportions of these different sequences varied from fraction to fraction. It was found that this variation in sequence was not due to coexistence of different proteins in the TBPII preparation, but rather reflected truncation in the same protein to varying degrees at its NH<sub>2</sub> terminus. Thus in the experiment shown in Fig. 2, NH<sub>2</sub>-terminal sequence analysis of the protein of fraction 27 revealed the sequence: Val-Ala-Phe-Thr-Pro-, but also, and in even higher amounts, a sequence shorter by three amino acids: Thr-Pro- and, in still higher amounts, a sequence which lacked just two of the terminal amino acids: Phe-Thr-Pro-. On the other hand, in

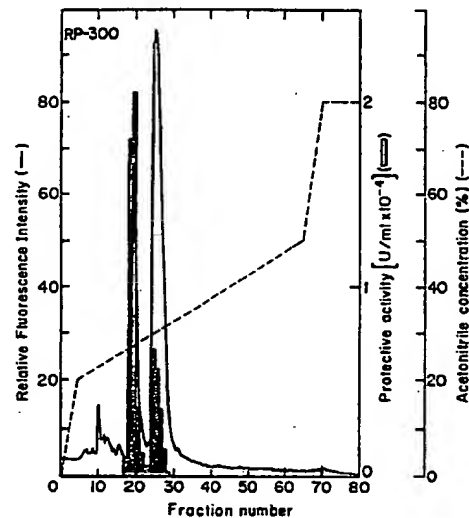


FIG. 3. Reversed phase HPLC of the TBPs. Ligand (TNF)-affinity column eluate was applied to an Aquapore RP300 column. Elution was performed with a gradient of acetonitrile in 0.3% aqueous trifluoroacetic acid (---) as described before (10). Fractions were examined for bioactivity (---) and protein (—) content.

TABLE II  
Binding of the two TNF-binding proteins to TNF- $\alpha$  and the effect of competitive cytokines

Proteins applied for competition for TBP binding <sup>a</sup>	Amounts of bound protein	
	<sup>125</sup> I-TBPI	<sup>125</sup> I-TBPII
	cpm	
rhuTNF- $\alpha$	27,700 ( $\pm$ 2,000)	17,634 ( $\pm$ 1,230)
rhuTNF- $\beta$	1,050 ( $\pm$ 140)	2,400 ( $\pm$ 174)
rIL-1 $\alpha$	21,000 ( $\pm$ 850)	6,240 ( $\pm$ 230)
rIL-6	28,100 ( $\pm$ 460)	17,840 ( $\pm$ 890)
rIFN- $\gamma$	27,050 ( $\pm$ 570)	18,570 ( $\pm$ 1,120)
TBPI	28,050 ( $\pm$ 1,050)	18,470 ( $\pm$ 1,430)
TBPII	1,900 ( $\pm$ 170)	2,240 ( $\pm$ 160)
	ND <sup>b</sup>	2,005 ( $\pm$ 150)

<sup>a</sup> All proteins were applied at a concentration of 10  $\mu$ g/ml.

<sup>b</sup> ND = not determined.

fraction 28, the sequence Val-Ala-Phe-Thr-Pro- was the major one; but, also in lower amounts the sequence Phe-Thr-Pro- was detected; and, in even lower amounts, the sequence Thr-Pro-.

To explore further the interrelations between TBPI and TBPII, we examined the extent of their immunological cross-reactivity. As shown in Fig. 4, in Western blotting analysis,

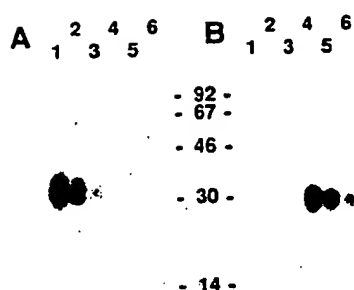


FIG. 4. Binding of TBPI and TBPII antisera to the TNF-binding proteins as analyzed by Western blotting. TBPI (A, lanes 1-6) and TBPII (B, lanes 1-6) were applied to SDS-PAGE at 2  $\mu$ g/lane, together with 2  $\mu$ g of BSA. Following electrophoresis, the proteins were blotted electrophoretically to a nitrocellulose sheet which was then incubated with antiserum to TBPI (lanes 1-3) or to TBPII (lanes 4-6) at the following dilutions: lanes 1 and 4, 1:100; lanes 2 and 5, 1:500; lanes 3 and 6, 1:2500. After incubation with the antibodies the nitrocellulose sheet was incubated with  $^{125}$ I-labeled protein A as described under "Materials and Methods."

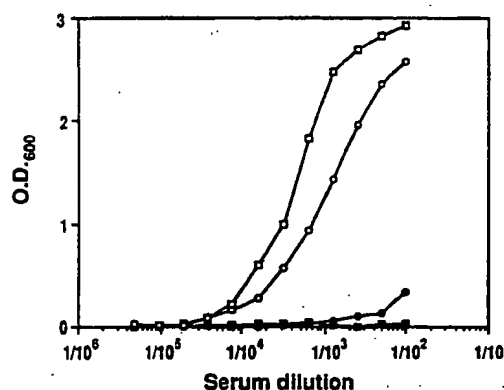


FIG. 5. ELISA, for the binding of antisera against TBPI and TBPII to the two species of TBP. The binding of  $\square$ , antiserum against TBPI to TBPI;  $\blacksquare$ , antiserum against TBPII to TBPII;  $\circ$ , antiserum against TBPII to TBPI; and  $\bullet$ , antiserum against TBPI to TBPI is presented in terms of the absorbance of the color product in the horseradish peroxidase assay. Control values (binding of the antisera to BSA) were subtracted from all readings.

antisera raised in rabbits against the two proteins recognized just that species of TBP against which they had been raised. Similarly, using ELISA, the antiserum against TBPI was found to react with TBPI at a dilution of up to 1:25,000, but did not react with TBPII even at a dilution of 1:100. Conversely, the antiserum raised against TBPII bound TBPII at up to a dilution of 1:25,000 (Fig. 5). In this sensitive and quantitative assay, some binding of the antiserum against TBPII to TBPI could be observed, but only to a small extent and at a low dilution of the serum (1:400). This binding was fully abolished when the antiserum was incubated, before immunoassay, with TBPI, but it was not affected by preincubation of the serum with TBPII (not shown). Our data, therefore, do not appear to reflect immunological cross-reactivity between TBPI and TBPII, but rather the presence of low amounts of antibodies to TBPI in the antiserum preparation, apparently due to the presence of some contaminating TBPI in the TBPII sample used for immunization. Such contamination is not unexpected, since in the final purification step of the TNF binding proteins the two proteins elute from the reversed phase HPLC column adjacent to each other (see below).

To examine the relation of the two urinary TNF-binding proteins to cell surface receptors for TNF, which according to recent evidence also exist as two distinct molecular species,<sup>2</sup> we tested the effect of the antisera to the two proteins on the binding of TNF to its receptors. Both antisera had a marked inhibitory effect (Fig. 6). Since the effect was observed in the cold (4 °C) and in the presence of sodium azide, which was applied to suppress metabolic activities in the cell, it clearly was not due to any induced change in expression of the TNF receptors. Rather it appears to reflect direct interaction of the antibodies with the receptors resulting in interference with the binding of TNF to them. The relative effectiveness of the antibodies against the two TBPs varied widely, depending on the cell type. In cells of the histiocytic lymphoma line, U937, the antiserum against TBPII had a marked inhibitory effect on TNF binding, while the antiserum to TBPI had no effect. In the chronic myeloid leukemia K562 cells, both antisera inhibited the binding of TNF, although to a different extent. Antibodies to TBPII abolished the binding completely while the antibodies to TBPI decreased TNF binding by only 40%, even when applied at high concentrations. In the HeLa, cervical carcinoma, and MCF 7 breast carcinoma cells, TNF binding was blocked effectively by the antiserum to TBPI; the antiserum to TBPII was also inhibitory, but only at much higher concentrations.

Since our quantitative study of the binding activities of the two antisera, using ELISA, revealed that the antiserum to TBPII contains also small amounts of antibodies to TBPI (Fig. 5), we preincubated samples of the antisera with either TBPI or TBPII, to determine whether, and to what extent, such contamination could account for the effects of the two antisera on TNF binding (Fig. 7). We found that 1) the inhibitory effect of the antiserum to TBPII on the binding of TNF to HeLa and MCF 7 cells could indeed be related to the effect of contaminating antibodies to TBPI in this antiserum. The inhibition was fully abolished when the antiserum against TBPII was preincubated with TBPI, but it was not affected by preincubation of the antiserum with TBPII at a concentration which abolished the effect of this antiserum on U937 cells. 2) The partial inhibition of TNF binding to the K562 cells by the antiserum to TBPI was due to a genuine effect of antibodies against this protein. It was not affected by preincubation of the antiserum with TBPII and was fully blocked by preincubation with TBPI. It thus appears that the K562 cells express both those receptor molecules which are recognized by antibodies to TBPII and, to a lesser extent, also receptors which are recognized by antibodies to TBPI.

These findings suggest that TBPI and TBPII display immunological cross-reactivity with the cell surface receptors for TNF. The findings are consistent with another recent study which suggests that the U937 and K562 cells express TNF receptors which are different from those expressed by the HeLa and MCF 7 cells<sup>2</sup> and indicate that there are cells (like K562) on which both kinds of receptors are expressed.

#### DISCUSSION

One of the two proteins whose purification is described in this study (TBPI) is identical to a TNF-binding protein we isolated previously from human urine by a chromatographic procedure (10). Purification of this protein has been described recently also by Olsson *et al.* (11), and it may also be identical to a "TNF inhibitor" purified from urine by Seckinger *et al.* (12). When isolated by a multistep chromatographic procedure, the TNF-binding protein turned out to be somewhat smaller than after ligand affinity purification (27,000 (10) compared to about 30,000). Yet in both cases the protein was

FIG. 6. Inhibition of the binding of TNF to different cell lines with antisera to TBPI (○) and TBPII (□). The net binding, observed in the absence of antisera (100%), was 2500 cpm in U937 cells, 1500 cpm in K562 cells, 2400 cpm in HeLa cells, and 1100 cpm in MCF7 cells.

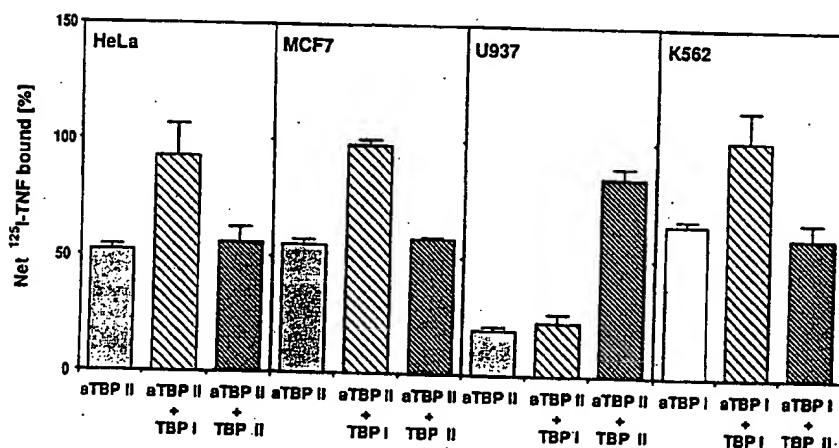
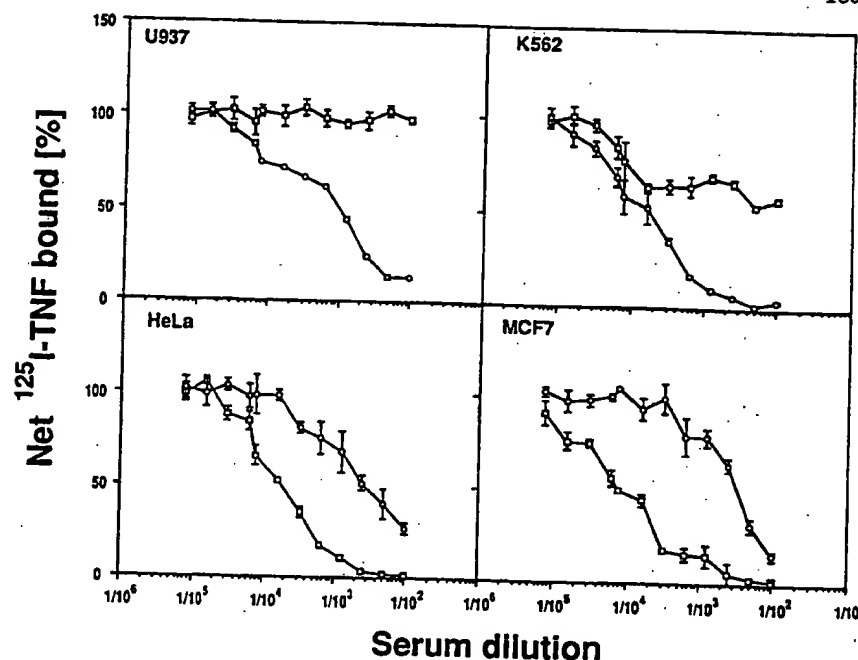


FIG. 7. Contribution of cross-contamination between the antisera to TBPI and TBPII to their effects on the binding of TNF to HeLa, MCF7, U937, and K562 cells. The antiserum to TBPI was applied at a dilution of 1:6000 and the antiserum to TBPII, at 1:400. The antisera were applied either alone or together with TBPI or TBPII at 1  $\mu$ g/ml. For other details, see "Materials and Methods." The net binding observed in the absence of antiserum was 2800 cpm in HeLa cells, 1100 cpm in the MCF7 cells, 2300 cpm in the U937 cells, and 1200 cpm in the K562 cells.

eluted from a reversed phase HPLC column at the same acetonitrile concentration and in both the same  $\text{NH}_2$ -terminal amino acid sequence was observed. A likely explanation for the difference in molecular size is the higher probability for proteolytic degradation in the more lengthy manipulations involved in the chromatographic purification as compared to affinity purification.

The second protein which we purified from human urine (TBPII) resembles TBPI in its molecular size, its ability to bind TNF, and its inhibition of the cytotoxic effect of TNF. However, TBPII can be clearly differentiated from TBPI by its lack of immunological cross-reactivity, differing  $\text{NH}_2$ -terminal amino sequences, and a difference in chromatographic properties which allowed its separation from TBPI by reverse phase HPLC. There are also indications that the two proteins are affected by proteases somewhat differently. As mentioned above, the molecular size of TBPI varies to some extent, depending on the purification procedure. This variation probably reflects degradation by proteases in the urine. However in spite of this variation in size the  $\text{NH}_2$ -terminal amino acid sequence of the protein remains unaltered implying that this degradation is restricted to its COOH terminus. On the other

hand, TBPII does show signs of degradation at the  $\text{NH}_2$  terminus, reflected in heterogeneity in the amino acid sequence.

Antibodies raised against the two urinary TNF-binding proteins inhibited the binding of TNF to cells. Consistent with recent evidence for existence of two species of TNF receptors whose relative proportion varies in different cell types,<sup>2</sup> antisera against the two proteins affected differentially the binding of TNF to different cells. These findings suggest immunological cross-reactivity and thus structural similarity between the urinary TNF-binding proteins and the cell surface receptors for TNF. Yet the molecular sizes of the soluble binding proteins are smaller than those reported for the cell surface receptors. The markedly differing extent to which TNF- $\alpha$  and TNF- $\beta$  were bound by the soluble proteins, as opposed to the similar extent of binding of those cytokines by the membranous TNF receptors, is also indicative of some structural differences between these proteins. Detailed analysis of the amino acid sequences of the soluble and membranous TNF-binding proteins should provide further information as to their structural relationship.

Some idea as to the mechanisms which underlie the exist-

ence of TNF-binding proteins in both cell surface and soluble forms can be derived from studies on other proteins found in such dual forms. Several mechanisms for such a phenomenon have been observed, each implying a differing extent of interdependence between the formation of the soluble and the membranous forms of the protein. Soluble and membranous proteins which are structurally related, yet coded by different genes, and thus able to be formed independently of each other are known (e.g. Ref. 17). There are also membranous and soluble forms of proteins in which both forms of the protein are coded by the same gene and yet are synthesized independently by translation of different species of mRNA produced via alternative splicing pathways. The soluble and cell-associated forms of the immunoglobulins provide such an example. Yet soluble and membranous forms of the same protein can also be formed in a tightly coupled manner, the soluble protein being produced by cleavage or shedding of the membranous one (e.g. Refs. 18 and 19). Structural studies of the soluble form of the  $\alpha$ -chain of the IL-2 receptor suggest that its formation involves such a step of proteolytic cleavage (20). Determining by what mechanism TBPI and TBPII are produced will contribute also to our understanding of the physiological function of these molecules. It is of particular interest to know whether the proteins are formed independently of the cell surface receptors or by their proteolytic cleavage, since in the latter case their formation should result in decreased amounts of the cellular receptors and thus may constitute a mechanism for a decrease in responsiveness of the cell to TNF.

Apart from the existence in the urine of what appears to be soluble forms of each of the two TNF receptors, probably derived from cells of different kinds, human urine has been found to contain also a soluble form of the  $\alpha$ -chain of the IL-2 receptor (21). Furthermore, we could detect soluble receptors in urine for IL-6 and IFN- $\gamma$  (22). There is probably a common underlying mechanism for the excretion of these different receptors. If this mechanism affects the receptors for other cytokines, it should be possible to isolate them also from urine. Indeed, there is limited evidence suggesting that receptors for several other cytokines are released by cells in a soluble form (e.g. 23-25). As our study shows, if these receptors are found to be excreted in the urine, their purification can be approached in a rather convenient way, simply by applying the crude urinary proteins on a column of the immobilized cytokine.

Another possibility raised by the findings in this study which may have practical importance is that receptors to cytokines, if presented to cells in a soluble form, may sequester the cytokine and thus serve as inhibitors. Detailed information on the structure of the receptors and knowledge of ways to produce them in a bioactive soluble form could provide us with inhibitory molecules which might act as therapeutic

agents for suppressing overresponse to these cytokines in disease.

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